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# GENOMIC ANALYSIS OF *EIMERIA* SPP. POPULATIONS IN RELATION TO PERFORMANCE LEVELS OF BROILER CHICKEN FARMS IN ARKANSAS AND NORTH CAROLINA

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ABSTRACT: The impact of coccidiosis outbreaks on the productivity of broiler chicken farms can be substantial, depending on the severity of disease caused by particular species and strains of *Eimeria*. We examined the genetic diversity of *Eimeria* species present in commercial broiler farms in relation to their performance level. Four groups of broiler chicken farms in Arkansas (AR) and North Carolina (NC), having either high or low performance levels, were sampled for *Eimeria* spp. oocysts. We amplified gDNA from oocysts by using genus-specific primers targeting 18S ribosomal RNA, the first and second internal transcribed spacer regions, and cytochrome c oxidase subunit I as the established species-specific primers. *Eimeria* spp. diversity was not homogenous among the 4 farm groups, with less-pathogenic species (*E. mitis* and *E. mivati*-like) associated with AR and NC high-performance farms, respectively, and a pathogenic species (*E. brunetti*) associated with AR low-performance farms. Sequence analyses identified multiple *E. maxima* and *E. mitis* genetic variants, from which 2 *E. maxima* variants were unique to low-performance farms. Distinct populations of sequences at the NC high-performance farms were identified as *E. mivati*-like, based on homology searches. Our study demonstrated the utility of analyzing multiple genomic loci to assess composition and polymorphisms of *Eimeria* spp. populations.

Globally, poultry farms are severely impacted by species of *Eimeria*, ubiquitous intracellular parasites that are the causative agents for coccidiosis disease. *Eimeria* spp. infect the gastrointestinal tract, where they undergo asexual and sexual development. Asexual replication of the parasites involves repeated host-cell invasions, which rupture the epithelial cells lining the intestine and cause enteritis. The ultimate result is a decrease in growth rate and a reduced ability to convert food to body mass, which correlates to a negative economic impact on the performance levels of chicken farms (Williams, 1998; Haug et al., 2008). Sexual reproduction in *Eimeria* spp. culminates in the production of oocysts that are excreted from the body in feces, enabling transmission to other naïve hosts via ingestion (Conway and McKenzie, 2007).

Seven species of *Eimeria* are commonly recognized that specifically parasitize chickens and cause avian coccidiosis. While all 7 species have been determined to negatively affect the health of the birds, the degree of pathogenicity is variable. *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, and *E. tenella* can cause moderate to severe morbidity including weight loss or impaired weight gain, diarrhea, dehydration, and mortality in severe cases of disease (Allen and Fetterer, 2002; Conway and McKenzie, 2007). Two species, *E. mitis* and *E. praecox*, are thought to be less pathogenic, yet studies have shown that they also negatively impact the growth and overall health of chickens (Gore and Long, 1982; Fitz-Coy and Edgar, 1992; Williams, 1998). The effect *Eimeria* spp. infections have on growth, overall health, and thus quality of birds raised at poultry facilities varies, but all species are thought to impact the economic performance of chicken farms. Chicken farms often harbor 2, or more, of these species at any given time (McDougald et al., 1986; Kučera, 1990; Morris et al., 2007), and it has been shown that individual chickens can be concurrently infected by multiple *Eimeria* spp. (Long and Joyner, 1984). Recent data support the hypothesis that such multi-species infections may alter the pathogenicity of the *Eimeria* spp. infecting chickens and affect the severity of disease (Haug et al., 2008; Jenkins et al., 2008). However, the correlation between specific species composition and genetic diversity of *Eimeria* spp. infecting poultry facilities, with the performance levels at these facilities, has yet to be addressed in the United States and has just begun to be addressed in Europe (Haug et al., 2008).

Anticoccidial drugs, along with biocontrol protocols, have been the predominate methods used to control clinical outbreaks of avian coccidiosis, yet the economic impact of sub-clinical *Eimeria* spp. infections is still costly (Allen and Fetterer, 2002). While anticoccidials remain useful in controlling avian coccidiosis, *Eimeria* spp. are developing resistance to the drugs currently in use (Chapman, 1994, 1998). The need to analyze genetic variation in *Eimeria* spp. is recognized as a pivotal factor in our ability to understand the dynamics of the distribution and control of coccidia, (Morris and Gasser, 2006) and remains to be determined.

Molecular techniques have been developed as reliable and specific methods to identify the species of *Eimeria* infecting chickens by isolating oocysts from fecal matter deposited on the litter of poultry facilities (Schnitzler et al., 1998, 1999; Lew et al., 2003; Jenkins et al., 2006a, 2006b). The foundation for these techniques makes use of species-specific variations encoded in the genomic DNA of *Eimeria* spp., to which complementary primers are designed to anneal and amplify these regions via polymerase chain reaction (PCR). Once amplified, these genomic fragments can be visualized, after electrophoretic separation in agarose or polyacrylamide gels, to ascertain the presence or absence of a particular *Eimeria* species targeted by the primers used. Further, these genomic fragments can be sequenced to provide insight into the genetic variation within and among populations of *Eimeria* species.

United States broiler (meat) chicken farms in Arkansas (n = 14) and North Carolina (n = 16), under similar management strategies and conventional anti-coccidial drug regimes, were qualitatively categorized according to their cost-of-production versus pound-of-meat-produced as a way to measure performance level. Equal numbers of farms categorized in this way as 'low performance' (high cost/lb) and 'high performance' (low cost/lb) were established as sampling sites to compare and contrast the composition of *Eimeria* spp. at these facilities. The purpose of the present study was to identify the composition of *Eimeria* spp. at these facilities and analyze their genetic variation in order to assess any correlations with performance levels at these farms.

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## METHODS

### Identification of chicken farm study sites

Broiler chicken farms in Arkansas (AR) and North Carolina (NC), managed under equivalent conditions using conventional drug rotation programs of ionophore, chemical/ionophore, and Maxiban/Monteban, were qualitatively classified using the ratio of production cost versus pound-of-meat-produced (cost/lb.). Farms having the lowest cost/lb. were classified as 'high performance,' and those having the highest cost/lb. were classified as 'low performance.' Equal numbers of 'high' and 'low' performance farm classifications in AR (7 each) and NC (8 each) were selected for this study. None of these farms were using *Eimeria* spp. oocyst vaccinations to combat coccidiosis.

### Collection and isolation of *Eimeria* spp. oocysts

Litter samples were collected from all farms in AR (14 total) and NC (16 total) during December 2006 and shipped overnight to the Animal Parasitic Diseases Laboratory, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland. All litter samples were processed within 1 mo after delivery using standard procedures (Ryley et al., 1976). Fecal droppings (~10 g each) from each litter sample were removed with forceps to 50-ml polypropylene tubes. The fecal droppings were hydrated by adding deionized water to achieve a final volume of 50 ml and then incubated for 16 hr on a rotating rocker at 4 C. The tubes were placed upright in a rack, the contents allowed to settle for 3 min, and 5 ml were removed to a 15-ml polypropylene tube containing 5 ml of 2 M sucrose. The oocyst–sucrose suspension was mixed by vortexing, then overlaid with 1 ml of deionized $H_2O$ and centrifuged at 2,500 *g* for 10 min at 4 C. After centrifugation, 1.5 ml of the top layer was removed and mixed with 13.5 ml $H_2O$ and then pelleted by centrifugation at 2,500 *g* for 10 min at 4 C. The oocyst concentration in each tube was estimated using a hemacytometer.

### Propagation of *Eimeria* spp. oocysts

We have previously shown that expansion of *Eimeria* spp. parasites in susceptible chickens is useful to increase the amount of starting material for gDNA isolation and has little effect on species composition (Jenkins et al., 2006a). Thus, oocysts recovered from litter, as described above, were inoculated per os into 2 groups of 2-wk-old coccidian-free (based on fecal sample analysis) chickens (n = 6 chickens/group) using an animal feeding–intubation needle (VWR International, Inc., Bridgeport, New Jersey) at ~$10^5$ oocysts per inoculum. Animals were housed in a clean animal building until fecal droppings were collected between days 5 and 10. Fecal samples were processed for total *Eimeria* spp. oocysts as described above.

### Isolation of gDNA from *Eimeria* spp. oocysts

*Eimeria* spp. oocysts (~$10^5$ total) were pelleted by centrifugation in a 1.5 ml-microcentrifuge tube for 5 min at 2,000 *g* and then treated with 200 µl of bleach for 10 min at room temperature. Oocysts were then washed with $H_2O$ 4 times, with 5 min of centrifugation at 2,000 *g*. The oocysts were re-suspended in 500 µl of buffer ATL (Qiagen Inc., Valencia, California), transferred to a 2-ml screw-cap tube (Fisher Scientific, Pittsburgh, Pennsylvania), and disrupted on a Mini-Bead Beater-8 Cell Disrupter (BioSpec Products, Inc., Bartlesville, Oklahoma) using 200 mg of sterile glass beads (~0.5-mm diameter) via 2, 2 min agitations, each followed by a 1-min incubation on ice. DNA was purified using QIAamp® DNA Mini Kit (Qiagen Inc.) according to manufacturer's protocol. DNA yield was quantified using a Beckman Coulter™ DU® 640 spectrophotometer (Beckman Instruments, Inc., Fullerton, California) and held temporarily at 4 C with storage at −20 C.

### PCR, cloning, and sequencing of COI, ITS-1/-2, and 18S rRNA from *Eimeria* spp.

The use of DNA primers to amplify the ITS1 region of specific *Eimeria* spp. has been previously described (Jenkins et al., 2006a); primers used in this study are given in Table I. Universal, genus-specific DNA primers targeting 3 genomic regions from *Eimeria* spp. (COI, ITS-1/-2, 18S rRNA) were designed by aligning sequences from each region for all chicken *Eimeria* spp. available in the GenBank® database (http://www.ncbi.nlm.nih.gov/) using ClustalX (Jeanmougin et al., 1998). Regions of highly conserved sequence were determined from the alignment, by eye, and used as priming sites. Nucleotide sequence, melting temperature (Tm), amplicon size, and annealing temperatures for these primers are given in Table I. The universal ITS-1/-2 primers are designed in the 18S rRNA (primer ITS-1) and 28S rRNA (primer ITS-2) genes, thus amplifying from the nuclear ribosomal gDNA the partial 18S rRNA, full-length ITS-1, 5.8S rRNA, and ITS-2, and the partial 28S rRNA loci. Approximately 810 bps of the COI mitochondrial gene, and 1,790 bps of the 18S rRNA nuclear gene, were amplified using the universal primers designed to target each of these genes (see Table I).

PCR was used to amplify targeted regions from purified gDNA using GoTaq® DNA Polymerase kit (Promega, Madison, Wisconsin). PCR products were visualized in 1% agarose gels using ethidium bromide staining. QIAquick gel extraction kit (Qiagen Inc.) was used to purify amplicons and these were cloned using pGEM®-T easy (Promega) or pCR®2.1 (Invitrogen, Carlsbad, California) vector system kits. Color screening identified recombinant clones, and the inserts from these were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) with vector priming sites (M13) and internal priming sites (18S rRNA only), and sequences were obtained using an ABI 3730 genetic analyzer (Applied Biosystems). Sequence data files were verified for quality and accuracy using Sequencher™ 4.8 software (Gene Codes Corporation, Ann Arbor, Michigan). Sequence identity of inserts was determined using MEGABLAST algorithm searches of the nucleotide database collection (nr/nt) via the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/). Sequences were screened for hybrid forms potentially generated during amplification via template jumping, and suspect sequences were eliminated from further analysis. To assess the level of genetic variability within and between *Eimeria* spp., PCR fragments amplified via 18S rRNA, COI, or ITS universal primer pairs were cloned and sequenced such that the nucleotide reads obtained were unambiguous. Primers were trimmed from the sequences to avoid potential bias in subsequent sequence analysis. To provide comparative values across all chicken *Eimeria* taxa, additional sequences were retrieved from GenBank for species from which we had little or no sequence data; these are included in Table III.

Seventy-one ITS-1/-2 ribosomal RNA region (FJ230311–FJ230381), 80 COI (FJ236380–236459), and 52 18S rRNA (FJ236329–FJ236379, FJ263947) unique sequences obtained from this study have been deposited in the GenBank database.

### Phylogenetic analyses

Phylogenetic analyses of *Eimeria* spp. ITS-1, ITS-2, 18S rRNA, and COI DNA sequences, isolated from the high- and low-performance farms in AR and NC, were performed using sequences aligned by using multiple sequence alignment software (MUSCLE) (Edgar, 2004) along with additional *Eimeria* spp. sequences obtained from GenBank. Comparable *Toxoplasma gondii* genomic sequences obtained from GenBank were also included in the alignments and used as outgroups in the phylogenetic analyses. Duplicate sequences within any given performance category were removed from the phylogenetic analyses to avoid redundancy. Sequences were trimmed at the same relative 5′ and 3′ ends, and alignments were imported to MEGA version 4 (Tamura et al., 2007) for phylogenetic analyses that included neighbor joining (NJ), minimum evolution (ME), and maximum parsimony (MP) methods. Robustness of phylogenies was assessed using bootstrap statistical tests.

## RESULTS

### Distribution of *Eimeria* spp.

Species-specific PCR amplification of the ITS-1 genomic region from oocyst gDNA resulted in the detection of *E. tenella*, *E. maxima*, *E. acervulina*, and *E. praecox* in common to both states and both performance categories (Fig. 1). *Eimeria mitis* and *E. brunetti* were additionally detected at both high- and low-performance farm categories in NC. Thus, a total of 6 species were detected at NC farms using species-specific primers. From

TABLE I. Primers used in this study.

| Target | Primer ID | Primer sequence (5′–3′) | Amplicon size (bp) | Annealing (°C) |
|---|---|---|---|---|
| Species-specific primers | | | | |
| *E. tenella* ITS1 | ETf* | AATTTAGTCCATCGCAACCCT | 278 | 55 |
| | ETr* | CGAGCGCTCTGCATACGACA | | |
| *E. necatrix* ITS1 | ENf* | TACATCCCAATCTTTGAATCG | 285 | 50 |
| | ENr* | GGCATACTAGCTTCGAGCAAC | | |
| *E. acervulina* ITS1 | EAf* | GGCTTGGATGATGTTTGCTG | 321 | 54 |
| | EAr* | CGAACGCAATAACACACGCT | | |
| *E. maxima* US ITS1 | EMfA1† | CWCACCACTCACAATGAGGCAC | 145 | 53 |
| | EMrA1† | GTGAWTCGTTYGRRAGTTTGC | | |
| *E. maxima* AUS ITS1 | EMfA2† | CGTTGTGAGAARACTGRAAGGG | 145 | 55 |
| | EMrA2† | GCGGTTTCATCATCCATCATCG | | |
| *E. praecox* ITS1 | EPf‡ | CATCATCGGAATGGCTTTTTGA | 368 | 50 |
| | EPr‡ | AATAAATAGCGCAAAATTAAGCA | | |
| *E. mitis* ITS1 | EMITf‡ | TATTTCCTGTCGTCGTCTCGC | 306 | 54 |
| | EMITr‡ | GTATGCAAGAGAGAATCGGGA | | |
| *E. brunetti* ITS1 | EBf* | GATCAGTTTGAGCAAACCTTCG | 311 | 55 |
| | EBr* | TGGTCTTCCGTACGTCGGAT | | |
| Universal primers | | | | |
| 18S rRNA | ERIB1§ | ACCTGGTTGATCCTGCCAG | ~1,790 | 57 |
| | ERIB10§ | CTTCCGCAGGTTCACCTACGG | | |
| COI | KM204§ | GTTTGGTTCAGGTGTTGGTTG | ~810 | 55 |
| | KM205§ | ATCCAATAACCGCACCAAGAG | | |
| ITS1-ITS2 | ITS-1§ | GGATGCAAAAGTCGTAACACGG | ~873 / ~1,010 | 52 |
| | ITS-2§ | TCCTCCGCTTAATAATATGC | | |

* Primers designed by Schnitzler et al. 1998.
† Primers designed by Lew et al. 2003.
‡ Primers designed by Schnitzler et al. 1999.
§ Primers designed in this study.

the AR samples, these primers detected *E. brunetti* uniquely from the low-performance farms, while *E. mitis* was uniquely detected from the high-performance farms. *Eimeria necatrix* was the only species not detected at any of the farms in NC or AR.

To detect *Eimeria* spp. from these chicken farms in an additional way, 12–36 clones from each of the 4 farm categories, for each of 3 genomic loci amplified using universal primers (Table I), were sequenced (Table II). The distribution of *Eimeria* spp. detected using the universal primers at all farm categories corroborate the species-specific primer results (Fig. 1), except in the following instances: (1) universal primers did not detect *E. mitis* from AR high-performance or from NC low-performance farms; (2) universal primers did not detect *E. acervulina* from AR high- or low-performance farms; (3) universal primers detected only *E. praecox* from AR low-performance farms; (4) universal primers did not detect *E. brunetti* from any farm category; and (5) the universal primers were able to detect *E. mivati*-like sequences, an 8th candidate *Eimeria* sp., from the NC high-performance farms. No species-specific primers were designed for *E. mivati*; therefore, no data are available for its species-specific amplification from the samples.

The ability of the universal primers to detect multiple *Eimeria* spp. varied dependant upon the primer pair used. Universal primer amplification of the COI and 18S rRNA regions detected 4 and 5 species, respectively, from the 4 farm categories (Table II). However, universal primer amplification of the ITS regions resulted in the detection of sequences from only 2 species, *E. maxima* and *E. mitis*. The proportional distribution of species amplified using the 3 sets of universal primers are given in Table II. Combined sequence data demonstrate *E. maxima* was the most abundantly amplified genome overall from each of the 4 farm categories, i.e., 72%, 67%, 37%, and 67%. *Eimeria tenella* sequences were the second-most abundantly detected from 3 categories (28%, 18%, and 29%), and at NC high-performance farms, both *E. tenella* and *E. mitis* sequences were second in abundance at 21% of the combined samples. *Eimeria acervulina* sequences were detected at low levels (4–6%) in the NC high- and low-performance samples only. *Eimeria praecox* (15%; AR low), *E. mitis* (21%; NC high), and *E. mivati*-like (16%; NC high) sequences were detected sporadically, each from only a single farm category.

## Genetic variability in the 18S rRNA and COI loci

Sequences from the 18S rRNA and COI genes of *Eimeria* spp., isolated from all high- and low-performing farms, were each combined and analyzed according to species, and results are tabulated in Table III. Cloning of amplicons from the 18S rRNA genomic region resulted in 53 recombinant clones from which full length, quality sequences were obtained. Five species of *Eimeria* were detected from these sequences (Table II), and the amplicons varied in size from 1,746–1,757 bps (Table III), with intra-specific genetic identity of all sequences ranging from a low of 97.2% within *E. maxima* sequences to a high of 99.9% within *E. acervulina* and *E. tenella* sequences. Identity scores calculated, using only sequences obtained from this study, are indicated in Table III with an asterisk. Amplification of the COI locus resulted in a total of 118 clones fully sequenced and analyzed.

| | *E.tenella* | | *E.mitis* | | *E.maxima* | | *E.acervulina* | | *E.praecox* | | *E.mivati* | | *E.necatrix* | | *E.brunetti* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Universal | Specific | Universal | Specific | Universal | Specific | Universal | Specific | Universal | Specific | Universal | Specific | Universal | Specific | Universal | Specific |
| AR High | + | + | - | + | + | + | - | + | - | + | - | nd | - | - | - | - |
| NC High | + | + | + | + | + | + | + | + | - | + | + | nd | - | - | - | + |
| AR Low | + | + | - | - | + | + | - | + | + | + | - | nd | - | - | - | + |
| NC Low | + | + | - | + | + | + | + | + | - | + | - | nd | - | - | - | + |

FIGURE 1. Detection of *Eimeria* species from 'high' and 'low' performance farms in Arkansas (AR) and North Carolina (NC) as per PCR analyses using universal versus species-specific primers. Presence (+), absence (−). Negative controls were run in tandem with all reactions. No species-specific primers were designed to detect *E. mivati* and, thus, there are no data (nd).

Four species of *Eimeria* were identified from the COI sequences having nearly identical size (766–767 bps) and with genetic identity ranging from a low of 94.1% within *E. maxima* to a high of 99.8% within both *E. maxima* and *E. tenella*. Taken together, 18S rRNA and the COI sequences are well conserved, with intraspecific identity in the 7 common chicken *Eimeria* species at ≥98.6%, with the exception of *E. maxima* having comparatively low identity at both the 18S rRNA (97.2%) and the COI (94.1%) loci.

## Genetic variability in the ITS loci

The ITS-1 + ITS-2 primer pair frequently resulted in the amplification of 2 distinct fragments, as detected after electrophoretic separation; a 'short' fragment of approximately 870 bps and a second 'long' fragment of approximately 1,010 bps. From these, 73 complete sequences were obtained and identified according to species (*E. maxima* or *E. mitis*) and to 'long' or 'short' form. The ITS-1 and ITS-2 regions from these sequences, although amplified together, were subsequently analyzed separately (Table III). The ITS-1 'long' form sequences from *E. maxima* ranged from 486 to 517 bps, with sequence identity ranging as low as 78.9%. The 'long' form sequences of *E. mitis* were longer (566–570 bps), but less variable, with identity from 98.5% to 99.4%. 'Short' form *E. maxima* ITS-1 sequences were between 363 and 368 bps with lowest identity at 90.4%, while 'short' form *E. mitis* ITS-1 sequences ranged from 432–444 bps with lowest identity score at 90.9%. Additionally, sequence and phylogenetic analyses (see below) of ITS-1 region sequences identified a third genetic form of *E. maxima* sequences that were between 476 and 477 bps with 95.5% to 97.2% identity.

Distinct variations in the size of *E. maxima* ITS-2 sequences, but not of *E. mitis* sequences, were apparent, although overlap did occur. 'Long' form ITS-2 *E. maxima* sequences were between 266 and 302 bps with 78.6–99.6% identity, while 'short' forms were between 275 and 286 bps with 88.4–99.6% identity. Comparison of the 'long' and 'short' form ITS sequences we isolated from both *E. maxima* and *E. mitis* reveal that there are 2 divergent genetic forms within each species. The *E. maxima* ITS-1 sequences are more divergent between the 'long' and 'short' forms than are the ITS-2 sequences, with 31.6%–37.8% and 53.3%–62.8% identity, respectively (Table III). Similarly, low overall identity occurred within ITS-1 of 'long' versus 'short' form sequences of *E. mitis*, from 33.4% to 34.6%. ITS-region sequences from additional *Eimeria* taxa were obtained from GenBank and analyzed, as given in Table III, for comparative purposes. In addition, we have included analysis of ITS-2 sequences from 3 ''operational taxonomic units'' of *Eimeria* recently described by Cantacessi et al. (2008), 2 of which (OTU 'X' and 'Y') had unusually wide ranges of identity scores, from as little as 60.9% (OTU 'X') and

TABLE II. Distribution of *Eimeria* species at high- and low-performance chicken farms from Arkansas (AR) and North Carolina (NC), as determined by PCR amplification of 3 genomic regions. Proportional values are given in parentheses. Sequences were assigned to species based on maximum identity scores from nucleotide BLAST (BLASTn) analysis (Altschul et al., 1990) against the nucleotide collection (nr/nt) database at NCBI.

| Location | Performance | COI | ITS-1/-2 | 18S rRNA | Combined |
|---|---|---|---|---|---|
| AR | High | n* = 36 | n = 25 | n = 2† | |
| | | *Ema* (0.31) | *Ema* (1.0) | *Ema* (1.0) | *Ema* (0.72) |
| | | *Ete* (0.69) | | | *Ete* (0.28) |
| AR | Low | n = 27 | n = 18 | n = 21 | |
| | | *Ema* (0.56) | *Ema* (1.0) | *Ema* (0.52) | *Ema* (0.67) |
| | | *Ete* (0.44) | | *Epr* (0.48) | *Epr* (0.15) |
| | | | | | *Ete* (0.18) |
| NC | High | n = 28 | n = 18 | n = 17 | |
| | | *Ema* (0.29) | *Ema* (0.33) | *Ema* (0.53) | *Ema* (0.37) |
| | | *Eac* (0.14) | *Emit* (0.67) | *Emiv* (0.41) | *Eac* (0.06) |
| | | *Ete* (0.46) | | *Emit* (0.06) | *Ete* (0.21) |
| | | *Emiv* (0.11) | | | *Emit* (0.21) |
| | | | | | *Emiv* (0.16) |
| NC | Low | n = 27 | n = 12 | n = 13 | |
| | | *Ema* (0.41) | *Ema* (1.0) | *Ema* (0.92) | *Ema* (0.67) |
| | | *Ete* (0.56) | | *Eac* (0.08) | *Eac* (0.04) |
| | | *Eac* (.04) | | | *Ete* (0.29) |

* n, number of clones sequenced. *E. maxima* (*Ema*), *E. acervulina* (*Eac*), *E. praecox* (*Epr*), *E. tenella* (*Ete*), *E. mitis* (*Emit*), *E. mivati*-like (*Emiv*).
† 11 clones from this category proved to be hybrid sequence artifacts and were eliminated from the study, hence the low sample size.

TABLE III. Genomic sequence variability in length and % identity among *Eimeria* sequences.

| | Species / form | Target† | Length (bp) | Intra-specific identity (%)‡ | Inter-specific identity (%) vs. 1 | vs. 2 | vs. 5 |
|---|---|---|---|---|---|---|---|
| 1 | *E. maxima* / 'long' form | ITS-1 | 486–517 | 78.9–99.7* | | | |
| | | ITS-2 | 266–302 | 78.6–99.6* | | | |
| 2 | *E. maxima* / 'short' form | ITS-1 | 363–368 | 90.4–99.7* | 31.6–37.8* | | |
| | | ITS-2 | 275–286 | 88.4–99.6* | 53.3–62.8* | | |
| 3 | *E. maxima* / '3rd' form | ITS-1 | 476–477 | 95.5–97.2* | 34.5–38.7* | 55.6–58.0* | |
| 4 | *E. maxima* | 18S rRNA | 1,746–1,751 | 97.2–99.8* | | | |
| | | COI | 766–767 | 94.1–99.8* | | | |
| 5 | *E. mitis* / 'long' form | ITS-1 | 566–570 | 98.5–99.4* | | | |
| 6 | *E. mitis* / 'short' form | ITS-1 | 432–444 | 90.9–99.7* | | | 33.4–34.6* |
| 7 | *E. mitis* | ITS-2 | 325–347 | 93.5–99.6* | | | |
| | | 18S rRNA | 1,749–1,755 | 98.8 | | | |
| 8 | *E. praecox* | ITS-1 | 484–485 | 98.9–99.5 | | | |
| | | ITS-2 | 382–414 | 86.8 | | | |
| | | 18S rRNA | 1,746–1,747 | 99.1–99.7* | | | |
| 9 | *E. mivati*-like | 18S rRNA | 1,747–1,748 | 98.1* | | | |
| | | COI | 766–767 | 96.4–99.3* | | | |
| 10 | *E. acervulina* | ITS-1 | 447–452 | 96.9–97.7 | | | |
| | | ITS-2 | 350–353 | 87.1–98.8 | | | |
| | | 18S rRNA | 1,748 | 99.2–99.9 | | | |
| | | COI | 766–767 | 98.6–99.7* | | | |
| 11 | *E. tenella* | ITS-1 | 416–523 | 78.2 | | | |
| | | ITS-2 | 481–486 | 96.5–99.5 | | | |
| | | 18S rRNA | 1,754–1,757 | 99.4–99.9 | | | |
| | | COI | 767 | 98.6–99.8* | | | |
| 12 | *E. necatrix* | ITS-1 | 485–563 | 79.1–97.7 | | | |
| | | ITS-2 | 449–501 | 84.1–93.6 | | | |
| | | 18S rRNA | 1,756 | 99.7 | | | |
| 13 | *E. brunetti* | ITS-1 | 490–492 | 96.3 | | | |
| | | ITS-2 | 361–386 | 96.4 | | | |
| 14 | OTU 'X' | ITS-2 | 283–298 | 60.9–90.8 | | | |
| 15 | OTU 'Y' | ITS-2 | 329–399 | 44.1–98.7 | | | |
| 16 | OTU 'Z' | ITS-2 | 389–402 | 76.5–94.9 | | | |

* Indicates sequences only from this study used in determining value.
† Primer regions have been excluded from these analyses.
‡ Identical sequences (100%) are not shown here.

44.1% (OTU 'Y'). As summarized in Table III, the ITS genomic regions are more polymorphic than the other loci we analyzed, i.e., 18S rRNA and COI.

## Phylogenetic analyses using the ITS loci

Representative trees from the phylogeny reconstructions of *Eimeria* spp. ITS-1 sequences (Fig. 2A) resulted in 10 well-defined clades with strong bootstrap support (86–100). *Eimeria praecox*, *E. brunetti*, *E. acervulina*, *E. tenella*, and *E. necatrix* each fell into a single clade. Three well-supported clades of *E. maxima* sequences were generated in accordance to the 'long', 'short', and intermediate 3rd size forms described above. Within the 'short' ITS-1 clade, 11 genetic variants from high performance AR farms, and 4 and 5 genetic variants from low-performance farms in AR and NC, respectively, were isolated. No 'short' form variants were isolated from any of the high performance NC farms. Sequences from all 4 farm categories fell within the *E. maxima* 'long' ITS-1 clade, with 11 and 5 genetic variants from high-performance, and 8 and 7 genetic variants from low-performance, farms in AR and NC, respectively. The intermediate 3rd-form clade of *E. maxima* was comprised solely of 3 genetic variants obtained from AR low-performance farms. ITS-1 *E. mitis* sequences, all isolated from NC high-performance farms, fell into 2 well-supported clades, also according to 'long' versus 'short' length forms. Three genetic variants occurred within the 'long' form clade and 9 genetic variants within the 'short' form clade.

Analysis of the ITS-2 sequences (Fig. 2B) resulted in 9 distinct clades (bootstrap = 67–100). *Eimeria mitis*, *E. praecox*, *E. acervulina*, *E. tenella*, and *E. necatrix* each formed a single, well-supported clade. The resolution of distinct 'long' and 'short' form clades for *E. mitis* ITS-2 sequences did not occur, as all genetic variants fell within a single, well-supported clade (bootstrap = 96). Similar to the results from ITS-1 analysis, 2 clades of *E. maxima* sequences were generated in accordance to the 'long' and 'short' sequence length forms; however, the 3rd intermediate-form clade of *E. maxima* was not distinguished using the ITS-2 sequences. Also, similar to results from the ITS-1 phylogenetic analysis, no 'short' form ITS-2 *E. maxima* sequences were isolated from NC high-performance farms. 'Short' form genetic variants numbered 12, 6, and 5 from AR high, AR low, and NC low, respectively. 'Long' form *E. maxima* ITS-2 sequences were isolated from all 4 farm categories, with 8 and 2 genetic variants from high performance and 9 and 6 genetic variants from low-

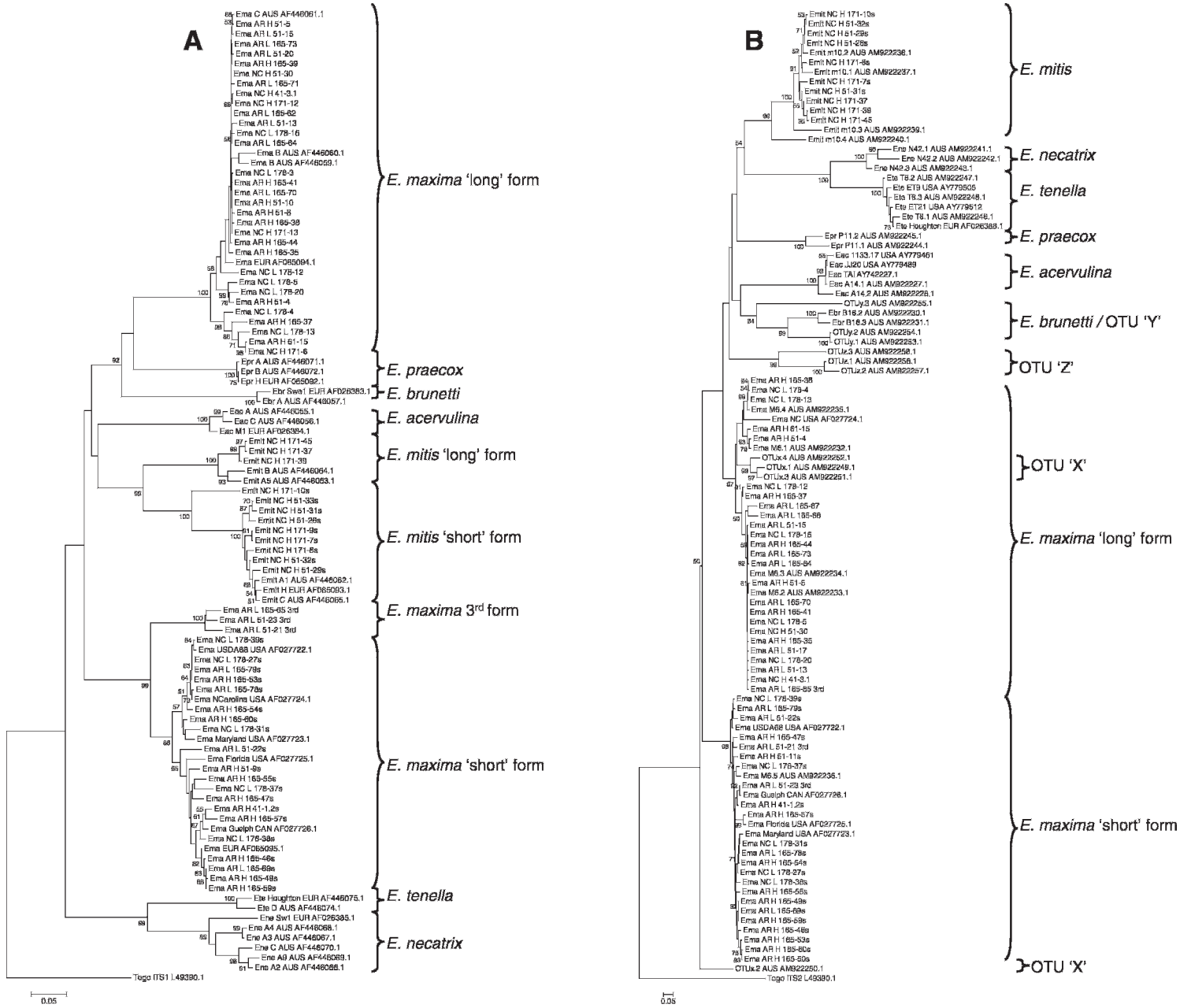


FIGURE 2. Phylogenetic analyses using the ITS-1 (**A**), ITS-2 (**B**), 18S rRNA (**C**), and COI (**D**) genomic regions amplified from *Eimeria* spp. The trees were generated by neighbor-joining distance-based method (Saitou and Nei, 1987), and robustness was tested using 1,000 rounds of bootstrapping (Felsenstein, 1985); only values ≥50 are shown. Scales reference branch lengths that are proportional to the evolutionary distance calculated using the maximum composite likelihood method (Tamura et al., 2004), with pairwise deletion of gaps, and are given in units of substitutions per site. Trees are rooted using *Toxoplasma gondii* sequences. Clones sequenced for this project are identified by state (AR or NC) and performance type (H = high; L = low). Identical sequences from the same location and performance level have been removed to avoid redundancy. Strain, geographic location, and accession number are given for database sequences used: AUS = Australia, CAN = Canada, CHN = China, EUR = Europe, TAI = Taiwan, USA = United States of America.

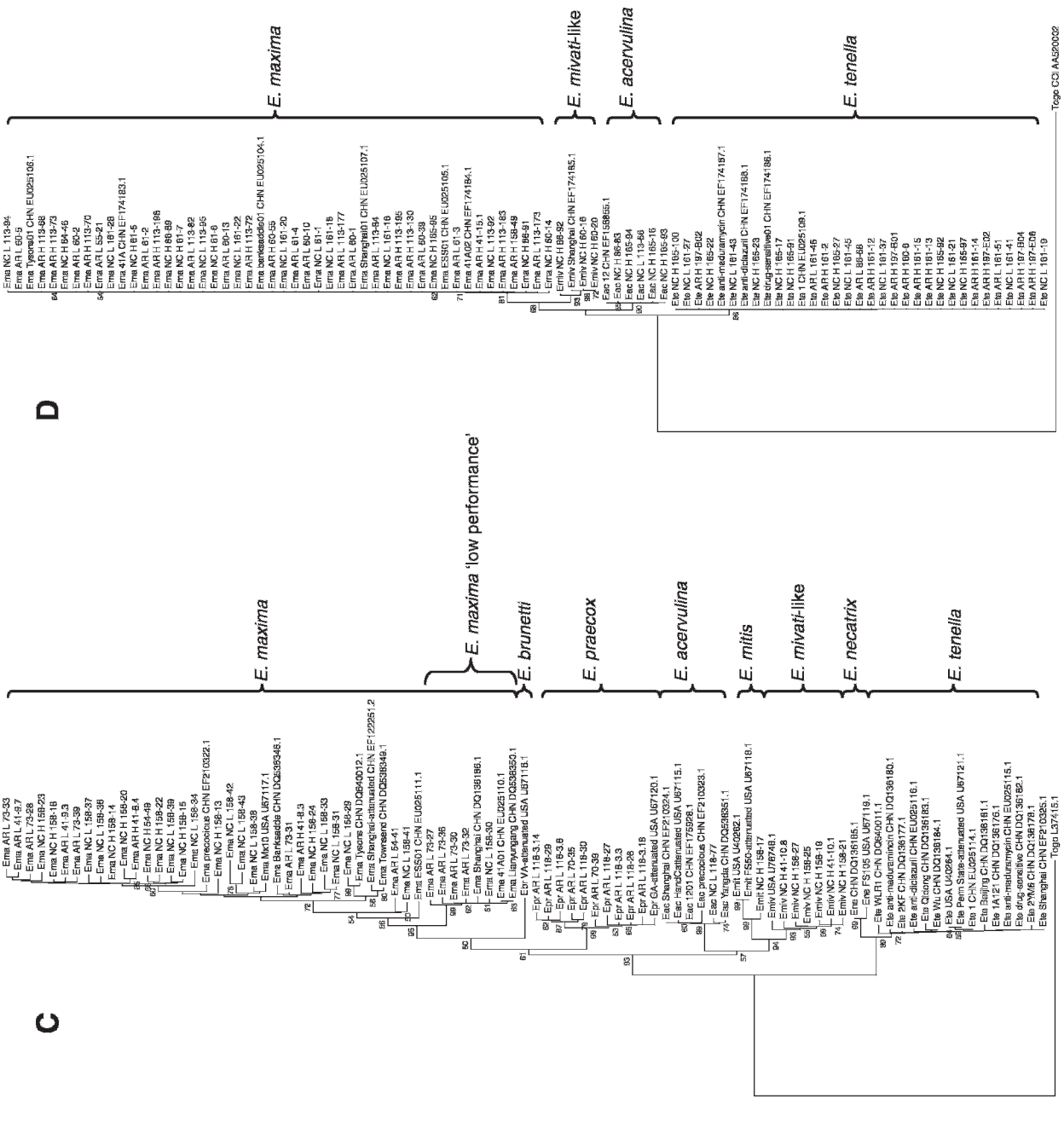


FIGURE 2. Continued.

performance farms isolated from AR and NC, respectively. The 'long' form *E. maxima* sequences included a well-supported subclade (bootstrap = 99) of 3 of the 4 OTU 'X' sequences isolated in AUS (OTUx.1, x.3, x.4), described as a 'population variant' of *E. maxima* (Cantacessi et al., 2008). None of the sequences we isolated from AR or NC clustered with this subclade. The 4th OTU 'X' sequence (OTUx.2) had an undetermined relationship to any of the other *Eimeria* spp. clades analyzed here. An 8th ITS-2 clade (bootstrap = 99) was comprised of 3 AUS sequences identified as OTU 'Z' by Cantacessi et al. (2008). The 9th clade of sequences (bootstrap = 84) included both *E. brunetti* and 3 AUS sequences identified as OTU 'Y' (Cantacessi et al., 2008). Two of the OTU 'Y' sequences (OTUy.1 and y.2) are sister to the *E. brunetti* sequences, with the 3rd OTUy.3 basal to these.

### Phylogenetic analyses of the 18S rRNA and COI loci

Phylogeny reconstruction using the 18S rRNA sequences (Fig. 2C) revealed species-specific clades (bootstrap = 72–99) that included a well-supported clade (bootstrap = 93) of 6 unique sequences isolated from high-performance farms in NC with the highest homology to an "*E. mivati*" sequence catalogued in GenBank (U76748.1). A large clade of sequences identified as *E. maxima* had the greatest abundance and diversity of 18S rRNA sequence variants, with 2 and 9 genetic variants from high-performance farms and 11 and 12 genetic variants from low-performance farms in AR and NC, respectively. Within the *E. maxima* clade, we identified a distinct subclade with strong support (bootstrap = 99) that was comprised of 5 sequence variants from low-performance farms in both AR (4 sequences) and NC (1 sequence), as well as 3 sequence variants isolated in China: (1) "Shanghai" (DQ136186.1); (2) "41A01" (EU025110.1); and (3) "Lianyungang" (DQ538350.1). Sequence variants of 18S rRNA had no obvious geographic correlations in the phylogenetic analyses.

COI had the greatest number of identical sequences obtained, with 38 clone sequences eliminated from the data set used to construct the phylogeny (Fig. 2D), leaving 80 sequences analyzed in total. Four species-specific clades occurred: *E. maxima* (bootstrap = 81), *E. acervulina* (bootstrap = 90), *E. tenella* (bootstrap = 66), and a distinct clade (bootstrap = 93) of 3 sequences isolated only from the NC high-performance farms that clustered with 1 other sequence obtained from GenBank, catalogued as *E. mivati* strain "Shanghai" from China (EF174185.1). Within the *E. maxima* clade, 11 and 8 genetic variants from high-performance and 14 and 9 variants from low-performance farms in AR and NC, respectively, were isolated. Within the *E. tenella* clade, we isolated 11 and 8 genetic variants from high-performance and 3 and 7 variants from low-performance farms in AR and NC, respectively. Four of the *E. acervulina* genetic variants isolated were from NC high-performance farms, while 1 variant occurred at the NC low-performance farms.

## DISCUSSION

In this study, we have gathered data to address whether the performance levels of broiler chicken farms, measured as cost-per-mass-produced, were correlated with the *Eimeria* spp. infecting the chickens at these facilities. Results of our species-specific PCR analyses, combined with sequence data obtained using universal *Eimeria* spp. primers, identified high levels of species diversity in oocyst DNA samples isolated from litter at farms having both high- and low-performance levels from Arkansas and North Carolina, although the specific species compositions were not homogenous (Fig. 1). High- and low-performance category farms in NC were all infected with 6 *Eimeria* species in common: *E. tenella*, *E. mitis*, *E. maxima*, *E. acervulina*, *E. praecox*, and *E. brunetti*. The NC high-performance farms were additionally infected with an *E. mivati*-like strain. Thus, the *Eimeria* species complexity was greater at the high-performance farms than at the low-performance farms in NC. This may be a contributing factor to the qualitative difference in performance levels, as the impact of coccidia infections on chickens may be ameliorated when additional species, or certain combinations of species, are present (Haug et al., 2008; Jenkins et al., 2008). Four species of *Eimeria* were detected in common at the AR high- and low-performance farms, i.e., *E. tenella*, *E. maxima*, *E. acervulina*, and *E. praecox*. However, *E. mitis* was uniquely present at the AR high-performance farms and *E. brunetti* was unique to the AR low-performance farms. The association of *E. brunetti* with the low-performance farms may be important, as it is known to be a significant pathogen of chickens. The presence of *E. mitis* at the high-performance farms may be important, as the impact of coccidiosis on chicken farm performance may be mitigated by the abundance or presence of less-pathogenic species (Haug et al., 2008).

Overall, the species-specific primers detected a greater diversity of *Eimeria* species at all 4 farm categories than did the universal primers. The universal primers were reliable at amplifying *E. tenella* and *E. maxima*; however, they did not efficiently detect *E. acervulina*, *E. mitis*, *E. praecox*, or *E. brunetti*. Still, the universal primers may be useful in the detection of non-traditional species variants, as exemplified by the amplification of sequences with the highest homology to *E. mivati*. The universal primers also identified distinct populations of genetic variants of *E. maxima* using sequence data from the ITS-2 (Fig. 2B) and 18S rRNA (Fig. 2C) genomic loci as well as populations of genetic variants from both *E. maxima* and *E. mitis* using ITS-1 data (Fig. 2A). As none of the samples was found to contain *E. necatrix*, the ability of the universal primers to detect this species remains undetermined. The species-specific primers used in this study (Table I) are well-established tools that specifically detect each of the 7 commonly recognized species of *Eimeria* pathogens known to infect chickens (Schnitzler, 1998, 1999; Lew et al., 2003). However, molecular analysis using non–species-specific oligonucleotide primers to amplify *Eimeria* spp. DNA, and the subsequent use of techniques such as the PCR-based capillary electrophoresis (CE) method (Woods et al., 2000; Gasser et al., 2005) and cloning and sequencing methods (as shown here), provide new ways of examining genetic diversity within *Eimeria* spp. of chickens. These techniques are now beginning to reveal genetic diversity within the *Eimeria* spp. infecting chickens (Morris et al., 2007; Cantacessi et al., 2008). However, the extent of this diversity has yet to be adequately described, the interrelationships of this diversity are yet to be interpreted, and the impact of this information on the management of chicken farms has yet to be applied.

In the present study, we have designed and used universal primers with the ability to detect multiple *Eimeria* spp. genotypes that may be present in any given sample. The overall number of *Eimeria* spp.

genetic variants we isolated at all 3 loci targeted with these primers (ITS-1/-2, 18S rRNA, and COI) were similar in number at high-performance (104 variants total; AR = 48, NC = 56), compared to low-performance (99 variants total; AR = 57, NC = 42), farms. Two populations of genetic variants, however, were specifically associated with only the low-performance farms we surveyed. First, from the 18S rRNA region sequences, a distinct subgroup of the *E. maxima* sequences, comprised of 5 genetic variants, formed a unique, well-supported clade (Fig. 2C). This distinct clade of sequence variants was associated only with low-performance farms in both AR and NC. Three sequence isolates from China also nested within this clade of *E. maxima* sequence variants, suggesting that this variant is geographically widespread. Second was a distinct clade comprised of 3 ITS-1 variants and identified as a 3rd form of the ITS-1 *E. maxima* sequences we isolated. While we isolated many genetic variants of *E. maxima* from low-performance farms in both AR and NC, this particular ITS-1 variant was only isolated from low-performance farms in AR. Whether populations of *E. maxima* containing these low-performance, farm-specific genotypes identified via the 18S rRNA and ITS-1 loci can be linked on a broader scale with performance levels at other chicken farms remains to be determined. While the population of 18S rRNA low-performance farm-associated variants we isolated included 3 GenBank-catalogued isolates from China, no information was available regarding the conditions of the chickens or farms from which those sequences were obtained. Using sequence polymorphisms unique to these genetic variants, primers can be designed to specifically survey oocysts isolated from litter samples via PCR analysis. In this way, a broad-scale survey of farms with qualified performance levels could be conducted to further research the association of these variants and their impact on chicken farm productivity.

In addition to the populations associated with low performance levels, we observed an overall high level of genetic diversity within the species identified from these farms. ITS-1 sequences revealed 2 well-supported populations of genetic variants of both *E. maxima* and *E. mitis*, described as 'long' and 'short' form populations, in addition to the 3rd form clade from *E. maxima* (Fig. 2A). Previously accessioned sequences from Australia, Europe, Canada, and the United States occurred among our sequences in the 'long' and 'short' form clades of *E. mitis* and *E. maxima*, though no other sequences were found to cluster with our 3rd form clade from *E. maxima*, suggesting that this form may be rare or restricted to our sampling locations. The 'long' and 'short' form specific clades from *E. maxima* were reflected in the ITS-2 analysis (Fig. 2B), though the *E. mitis* sequences were collapsed into a single clade and the *E. maxima* 3rd form clade was lost, with sequences split among the 'long' and 'short' form *E. maxima* ITS-2 clades. Recently described *Eimeria* spp. operational taxonomic units (OTUs) X, Y, and Z, by Cantacessi et al. (2008), were included in our phylogenetic analyses of the ITS-2 region sequences (Fig. 2B). Three of the OTU 'X' sequences were well supported within our *E. maxima* 'long' form population of genetic variants. A 4th OTU 'X' sequence ('OTUx.2' AM922250.1) was highly divergent, with no clear association to any other *Eimeria* spp. in our analysis, and its status remains puzzling. The OTU 'Y' variants clustered with the *E. brunetti* sequences and may be a glimpse into some of the genetic diversity present within this species. The OTU 'Z' variants formed a unique clade within the *Eimeria* spp. in our analysis and may prove to be a novel population.

As we have shown here, the use of universal *Eimeria* spp. primers targeting multiple loci are valuable tools in the identification of genetic variants within the populations infecting chickens. While most studies of genetic diversity in chicken *Eimeria* spp. analyze either ITS-2 (most) or ITS-1, our analyses of sequences obtained from both ITS-1 and ITS-2 regions (using primer ITS-1 and ITS-2, Table I) provided better insight into the genetic diversity from our samples than we would have gleaned using only 1 of these regions (Figs. 2A, B). Our data from 18S rRNA sequences provided additional insight to our study, identifying sequences from the greatest number of *Eimeria* species, i.e., 5 (Table II), as well as enabling the identification of a distinct population of *E. maxima* associated with low-performance farms (Fig. 2C). In addition, phylogenetic analyses of 18S rRNA and COI sequences isolated from the farms we surveyed each revealed distinct clades comprised of sequence variants that did not cluster with any other species and had the greatest similarity to sequences catalogued in GenBank as a putative 8th chicken *Eimeria* spp., *E. mivati*. While the validity of *E. mivati* as a distinct species remains debated, our analyses demonstrate these sequences have enough polymorphisms in both the 18S rRNA nuclear genomic locus and the COI mitochondrial locus to form a distinct *Eimeria* spp. clade. In the 18S rRNA tree (Fig. 2C), the *E. mivati*-like sequences are most similar to *E. mitis* sequences, with strong support (bootstrap = 94). The relationship between *E. mitis* and the *E. mivati*-like COI sequences we isolated could not be determined, as no COI sequences from *E. mitis* were available. Thus, the universal primers for 18S rRNA and COI (Table I) should be useful in future studies to identify *E. mivati*-like sequences in chicken farm surveys and may help to establish the prevalence, distribution, and validity of this putative species.

As chicken *Eimeria* spp. sequences continue to be accessioned in publicly available databases, it is important for researchers to include as much information as possible regarding the status under which the sequences they choose to catalogue were isolated. With this information available, it is likely that particular genetic polymorphisms can be linked with characteristics such as pathogenicity, rate of life-cycle development, drug resistance, and overall performance level of chicken farms.

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